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Diacetyl Biosynthesis in *Streptococcus diacetilactis* and *Leuconostoc citrovorum*

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Pyruvate was shown to be the precursor of diacetyl and acetoin in *Streptococcus diacetilactis*, but dialyzed cell-free extracts of *S. diacetilactis* and *Leuconostoc citrovorum* that had been treated with anion-exchange resin to remove coenzyme A (CoA) formed only acetoin from pyruvate in the presence of thiamine pyrophosphate (TPP) and Mg^{++} or Mn^{++} ions. The ability to produce diacetyl was restored by the addition of acetyl-CoA. Acetyl-phosphate did not replace the acetyl-CoA. Neither diacetyl nor acetoin was formed when the otherwise complete reaction system was modified by using boiled extract or by omitting the extract, pyruvate, TPP, or the metal ions. Free acetaldehyde was not involved in the biosynthesis of diacetyl or acetoin from pyruvate, dialyzed cell-free extracts of the bacteria produced only acetoin (besides CO_2) from α-acetolactate, and acetoin was not involved in the biosynthesis of diacetyl. Only one of the optical isomers present in racemic α-acetolactate was attacked by the extracts, and there was no appreciable spontaneous decarboxylation of the α-acetolactate at the *p*H (4.5) used in experiments.

The mechanism by which citrate-fermenting bacteria form diacetyl has not been established, although this volatile compound has been recognized since 1929 as an important food-flavor component (19). Historically, it has been assumed that acetoin (3-hydroxy-2-butanone) is the immediate precursor of diacetyl (2,3-butanedione). Sebek and Randles (22) reported that *Pseudomonas fluorescens* formed diacetyl when 2,3-butanediol was the only source of carbon, and they supported a pathway involving successive oxidations from 2,3-butanediol to acetoin to diacetyl. This oxidation of acetoin to diacetyl was considered appropriate for cultured dairy products as recently as 1966 (12), though Michaelian and Hammer (17), Cox (1), and Pette (20) had found lactic cultures unable to convert acetoin to diacetyl under a variety of experimental conditions, and Strecker and Harary (26) had found *Aerobacter aerogenes* and *Staphylococcus aureus* unable to form diacetyl from acetoin.

The biosynthesis of acetoin in bacteria has been shown by Juni (8) to proceed by enzymatic condensation of two molecules of pyruvate to yield one of CO_2 and one of α-acetolactate. The reaction requires thiamine pyrophosphate (TPP) and manganous or magnesium ions. In a subsequent reaction, the α-acetolactate is decarboxylated to give acetoin and CO_2. This α-acetolactate is optically active, which distinguishes it from the racemic α-acetolactate that is formed by pyruvate oxidase preparations (10).

De Ley (13) reported that some species of *Acetobacter* can form acetoin from pyruvate and free acetaldehyde in reactions that are analogous to the mechanism that occurs in yeasts and mammalian tissues, but studies with other bacteria indicate that free acetaldehyde is not involved in their synthesis of acetoin (9, 11, 14).

Some bacteria can form acetoin by reducing diacetyl. This conversion of diacetyl to acetoin by diacetyl reductase has been reported to be both reversible (22) and irreversible (1, 17, 23, 27).

After α-acetolactate was established as an intermediate in the formation of acetoin (8), there were conflicting reports as to the possibility that diacetyl can be formed in addition to acetoin in the decarboxylation of α-acetolactate. Dolin and Gunsalus (3) found that *Streptococcus faecalis* did not yield diacetyl, acetaldehyde, or lactate from α-acetolactate, but de Man (16) reported that ferric, cupric, or aluminum ions catalyze a nonenzymatic liberation of diacetyl from α-acetolactate. Mizuno and Jezeski (18) and Seitz et al. (E. W. Seitz, Ph.D. Thesis, Oregon State Univ., Corvallis, 1962; and 23) have reported that cell-free extracts of starter bacteria can decarboxylate α-acetolactate.

This study was undertaken to evaluate the mechanisms indicated in the literature and to de-

termine their relationships to diacetyl biosynthesis in *Streptococcus diacetilactis* and *Leuconostoc citrovorum*. Until recently, research on the biosynthesis of diacetyl has been hampered by the use of complex test systems and methods inadequate for quantitative separation and determination of the microgram quantities of diacetyl and acetoin found in cell-free test systems. This problem is resolved by the column salting-out chromatographic method we developed (25) for separating diacetyl, acetoin, and 2,3-butylene glycol.

The present study has disclosed a new mechanism that accounts for the biosynthesis of diacetyl. A possible role of free acetaldehyde in the synthesis of diacetyl and acetoin by *S. diacetilactis* was eliminated. The results show the two mechanisms by which these bacteria form acetoin, and reveal that neither α-acetolactate nor acetoin is a precursor of diacetyl in these bacteria.

MATERIALS AND METHODS

S. diacetilactis 18-16 and *L. citrovorum* CAF_1 were propagated routinely in sterile litmus milk. Cell-free extracts were prepared as follows. Cells of *S. diacetilactis* were grown for 16 hr (*L. citrovorum*, 36 hr) at 22 C, without shaking, in citrate broth at *p*H 6.6 (5), harvested by centrifugation, washed twice with cold 1 N tris(hydroxymethyl) aminomethane-cysteine (Tris-cysteine) buffer at *p*H 7.3 (0.001 M with respect to L-cysteine), and resuspended in the same buffer at a cell density of ca. 50 mg (dry weight) per ml. Preparative operations with the cell-free extracts were performed in a refrigerated room maintained at 4 C except where indicated otherwise. Cells were disrupted by ballistic disintegration with a Mickle tissue disintegrator operated at maximal amplitude for 45 min at 0 C. After centrifugation for 30 min at 2 C in a refrigerated centrifuge at 15,000 × *g*, the cell-free supernatant fluid was dialyzed for 24 hr against 100 volumes of 1 N Tris-cysteine buffer, at *p*H 7.3. For certain indicated experiments, coenzyme A was removed from dialyzed extracts by treating them with an anion-exchange resin (acid-washed Dowex 1-X2). One volume of the extract was stirred slowly for 5 min with one-quarter volume of the resin. The *p*H was checked periodically and maintained between 7 and 7.3 by dropwise addition of 1 N Tris-cysteine buffer. The resin was sedimented by centrifugation for 3 min at 5,000 × *g*, and the supernatant fluid was removed with a Pasteur pipette. One μmole of ethylenediaminetetraacetate (EDTA) per mg of extract protein was added to the supernatant fluid to remove metal ions.

Protein was determined by the biuret method (4) or the method of Lowry et al. (15). Acetoin and diacetyl were separated by the method of Speckman and Collins (25) and determined quantitatively by the Westerfeld method (28).

Sodium pyruvate, thiamine pyrophosphate (TPP), oxidized and reduced nicotinamide adenine dinucleotide (NAD, $NADH_2$), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$), acetyl-phosphate, acetyl-coenzyme A (acetyl-CoA), and sodium pyruvate-*3*-^{14}C (specific activity, 4.6 × 10^4 counts per min per μmole) were obtained from Calbiochem (Los Angeles, Calif.). Acetyl-CoA-*1*-^{14}C (specific activity, 25 mc/mmole) was obtained from New England Nuclear Corp. (Boston, Mass.). Isotopically labeled acetoin (specific activity, 5.9 × 10^4 counts per min per μmole) was prepared by the action of cell-free extracts of *S. diacetilactis* 18-16 on sodium pyruvate-*3*-^{14}C. The labeled acetoin was recovered and purified by salting-out chromatography. Diacetyl, acetaldehyde, and acetoin were obtained from Eastman Organic Chemicals Co. Acetoin, obtained as the crystalline dimer, was washed with ether until free from diacetyl as shown by both column and gas chromatography. Esterified α-acetolactate was obtained from K & K Laboratories (Jamaica, N.Y.). An infrared spectrum determined for the compound received from K & K (Fig. 1) matched the published spectrum for the acetoxy ethyl ester of α-acetolactate (21). α-Acetolactate was prepared from the ester by saponifying with two equivalents of cold 0.025 N NaOH added slowly to the system held in a water bath at 5 C and continuously purged with a stream of nitrogen.

All spectrophotometric measurements were made with a Beckman spectrophotometer, model DB. A Klett-Summerson colorimeter, model 800-3 with filter no. 54, was used for Westerfeld analyses. The *p*H was measured with a Radiometer *p*H meter, model 22 (Copenhagen, Denmark). Effluent was collected and distributed during chromatographic separations with a Radi-Rak fraction collector (Stockholm, Sweden). Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer with 0.3% 2,5-diphenyloxazole (PPO) and 0.01% 1,4-bis-2-(5 phenyl-oxazolyl)-benzene (POPOP) in a total volume of 20 ml of toluene.

RESULTS

Conversion of pyruvate to acetoin and diacetyl. A quantity of cell-free extract of *S. diacetilactis* was held in a water bath at 25 C with sodium pyruvate-*3*-^{14}C and cofactors as indicated in Fig. 2. After 30 min, a sample of the reaction mixture was removed and the acetoin and diacetyl

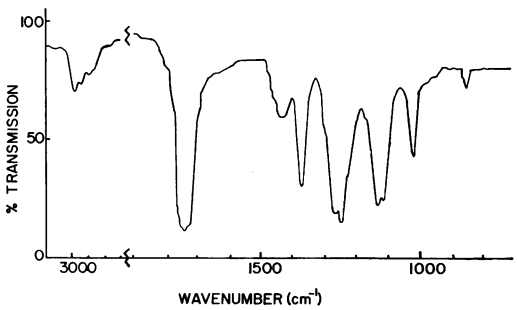

FIG. 1. *Thin-film infrared absorption spectrum of commercial esterified α-acetolactate.*

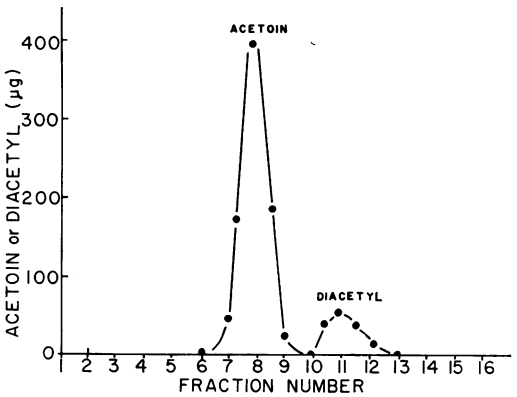

FIG. 2. *Conversion of pyruvate to acetoin and diacetyl by a cell-free extract of Streptococcus diacetilactis. The reaction mixture contained: Na-pyruvate-3-^{14}C, 100 μmoles (specific activity 4.6 × 10^4 counts per min per μmole); TPP, 0.02 μmole; $MgSO_4$, 4.5 μmoles; extract protein, 3 mg; 0.1 M phosphate buffer, pH 4.5; total volume, 3.0 ml; temperature, 25 C.*

were separated, determined quantitatively, and assayed for radioactivity. Each was radioactive, indicating that pyruvate had served as its precursor. Specific activity of the acetoin was 5.9 × 10^4 counts per min per μmole; specific activity of the diacetyl was 6.2 × 10^4 counts per min per μmole.

Free acetaldehyde not involved in the formation of acetoin or diacetyl. A dialyzed cell-free extract of *S. diacetilactis* 18–16 was used to determine whether this organism can use free acetaldehyde in the biosynthesis of acetoin or diacetyl. Reaction mixtures containing 3 mg of extract protein, 100 μmoles of pyruvate, 0.025 μmole of TPP, and 4.5 μmoles of Mg^{++} (with and without 100 μmoles of acetaldehyde) in 0.1 M KH_2PO_4 buffer, *p*H 4.5, were held 2 hr in a water bath at 25 C. Determination of the reaction products revealed that 42.1 μmoles of acetoin and 4.8 μmoles of diacetyl were formed in the presence of acetaldehyde, and that 41.7 μmoles of acetoin and 5.9 μmoles of diacetyl were formed when no acetaldehyde was added.

Additional evidence that free acetaldehyde is not involved in the formation of acetoin or diacetyl from pyruvate was obtained in the above experiment by adding 500 μmoles of Na_2SO_3 to a reaction mixture (without added acetaldehyde) to bind any acetaldehyde formed from the pyruvate. The cell-free extract produced 41.5 μmoles of acetoin and 5.6 μmoles of diacetyl in the presence of the Na_2SO_3, though it is known that excess Na_2SO_3, by binding free acetaldehyde, completely inhibits the formation of acetoin in yeasts (27).

We confirmed the reported influences of acetaldehyde and Na_2SO_3 on acetoin formation in yeasts by using a cell-free extract of *Saccharomyces cerevisiae*. Reaction mixtures contained 3 mg of extract protein, 25 μmoles of pyruvate, 0.025 μmole of TPP, and 4.5 μmoles of Mg^{++}, with and without 125 μmoles of Na_2SO_3 and with and without 25 μmoles of acetaldehyde. Reaction mixtures, in 0.1 M KH_2PO_4 at *p*H 4.5, were held 2 hr in a water bath at 25 C. The extract produced 180 μg of acetoin without Na_2SO_3 and only 9 μg with Na_2SO_3 added. The addition of acetaldehyde stimulated the production of acetoin; with acetaldehyde added, there was 227 μg of acetoin.

Acetoin from decarboxylation of α-acetolactate. Dialyzed cell-free extracts of *S. diacetilactis* and *L. citrovorum* were held in a water bath at 25 C with α-acetolactate as indicated in Fig. 3, samples were removed, and the products were separated and determined quantitatively. The *p*H for the experiments was 4.5 because these bacteria nor-

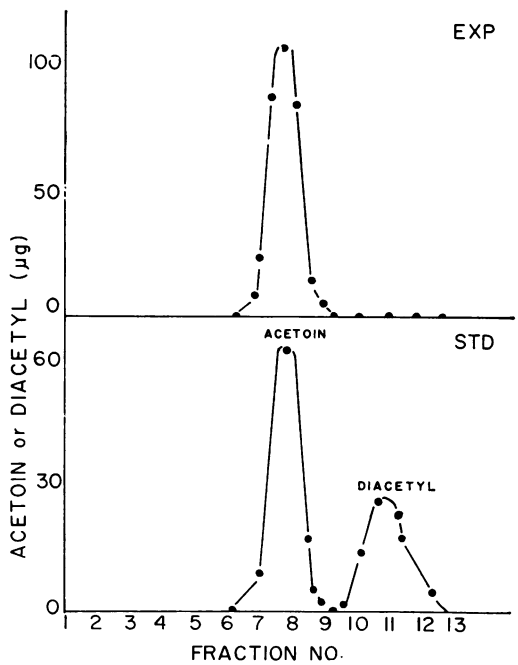

FIG. 3. *Decarboxylation of α-acetolactate by dialyzed cell-free extracts of Streptococcus diacetilactis and Leuconostoc citrovorum. Reaction mixtures contained: α-acetolactate, 10 μmoles; $MgSO_4$, 4.5 μmoles; extract protein, 5 mg; 0.1 M phosphate buffer, pH 4.5; total volume, 3 ml. The reaction time was 15 min at 25 C. Acetoin and diacetyl in the reaction mixtures were separated by salting-out chromatography and were determined quantitatively. STD is a standard elution graph of a mixture of acetoin and diacetyl (100 μg of each). EXP is an elution graph of the experimental reaction mixture.*

mally produce much larger amounts of diacetyl at low *p*H.

Decarboxylation of α-acetolactate by the extracts resulted in the formation of acetoin, substantiating the finding of Juni (8) that α-acetolactate is an important precursor of acetoin in bacteria. Although we found α-acetolactate to decarboxylate spontaneously under prolonged acidic conditions (about 5% per hr at 25 C), results showed no significant nonenzymatic decarboxylation in our experiments, in which the reaction times were usually 15 min and not longer than 30 min. From 10 μmoles of α-acetolactate, only 4.8 μmoles of acetoin was formed. Addition of 3 N H_2SO_4 resulted in decarboxylation of the remaining α-acetolactate, indicating that the enzyme can attack only one of the optical isomers present in racemic α-acetolactate.

Only acetoin was formed by *S. diacetilactis* or *L. citrovorum* in the enzymatic decarboxylation of α-acetolactate. There was no detectable diacetyl. This finding of acetoin as the only end product (other than CO_2) was not altered by adding the following cofactors to the test system given in Fig. 3: FAD, NAD, FAD plus NAD, $NADPH_2$, FMN, or FMN plus NAD. The substitution of ferric ions for the magnesium ions in equimolar amounts did not change the products of α-acetolactate decarboxylation.

Acetoin not involved in the formation of diacetyl. Dialyzed cell-free extracts of *S. diacetilactis* and *L. citrovorum* were held in a water bath at 25 C with diacetyl and cofactors as indicated in Fig. 4, samples of the reaction mixtures were removed, and the products were separated and determined quantitatively. The conversion of diacetyl to acetoin was enzymatic and dependent on the presence of $NADH_2$. Results for each of the organisms showed that acetoin is the product of this diacetyl reductase reaction and that diacetyl is in fact reduced. The results for *S. diacetilactis* are in Fig. 4. It was important to show that diacetyl is reduced, because we have found both *S. diacetilactis* and *L. citrovorum* to have $NADH_2$ oxidase activity similar to that reported by Dolin (2) for *S. faecalis*. This $NADH_2$ oxidase activity was found to interfere with spectrophotometric analysis of $NADH_2$ oxidation as a measure of diacetyl reductase activity. Its presence accounts for the fact that only about 85% of the diacetyl was converted to acetoin (Fig. 4).

Results indicated that the reaction catalyzed by diacetyl reductase is not reversible and that acetoin is not involved in the biosynthesis of diacetyl. No diacetyl was formed when acetoin was substituted for diacetyl in the test system described for Fig. 4, with or without the substitution of NAD for $NADH_2$. Results were the same

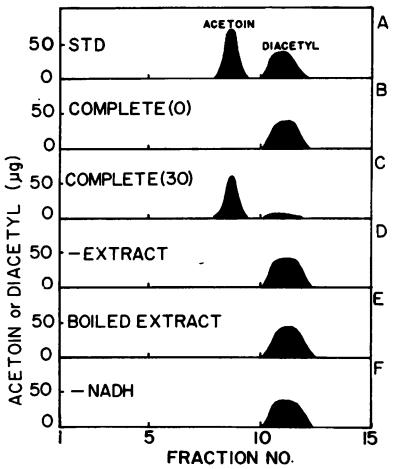

FIG. 4. *Conversion of diacetyl to acetoin by the diacetyl reductase of Streptococcus diacetilactis. Complete reaction mixture contained: diacetyl, 1 μmole; $NADH_2$, 1 μmole; 0.05 M phosphate buffer, pH 6.2; extract protein, 3 mg; total volume, 3 ml; temperature, 25 C. (A) Standard elution graph of a mixture of acetoin and diacetyl (100 μg of each). (B to F) Elution graphs of the complete reaction mixture and of the complete mixture modified as indicated on the graphs. Reaction times (in minutes) are indicated in parentheses.*

for both organisms over the *p*H range of 4.5 to 8.1.

Additional evidence was obtained for *S. diacetalactis* in two different experiments involving radioactive compounds. The test system for one experiment contained 0.12 μmole of ^{14}C-labeled acetoin, 100 μmoles of unlabeled sodium pyruvate, 0.025 μmole of TPP, 4.5 μmoles of Mg^{++} ions, 10 μmoles of NAD, and 3 mg of extract protein, all in 0.1 M KH_2PO_4 buffer at *p*H 4.5. The mixture was held for 30 min at 25 C, and a sample was removed for separation of acetoin and diacetyl and examination of the diacetyl for radioactivity. The resulting diacetyl was not radioactive, although it had been formed in the presence of radioactive acetoin.

In a different experiment, 50 μmoles of unlabeled acetoin was added to a mixture of sodium pyruvate-3-^{14}C, extract, and cofactors (similar to those indicated in Fig. 2) to determine whether or not the unlabeled acetoin would result in dilution of the radioactivity of the diacetyl formed from the pyruvate. It did not. The specific activity of the diacetyl formed in the presence of the added unlabeled acetoin was 7.4×10^4 counts per min per μmole compared to 6.2×10^4 counts per min per μmole for diacetyl that was formed in the absence of added acetoin.

Mechanism of diacetyl biosynthesis. The organ-

isms obviously formed diacetyl by a mechanism different from that used in the formation of acetoin. Consideration was given to mechanisms involving TPP in α-keto acid decarboxylations and to the apparent mechanism by which acetoin is formed via α-acetolactate. It seemed that condensation of the acetaldehyde-TPP complex with a compound having an acetyl-ester linkage might allow the necessary migration of electrons to yield diacetyl rather than acetoin. Consequently, we decided to determine whether acetyl-CoA, with its thiol-ester linkage, is involved in the biosynthesis of diacetyl.

Coenzyme A was removed from dialyzed cell-free extracts of *S. diacetilactis* and *L. citrovorum* with anion-exchange resin (acid-washed Dowex 1-X2). In the presence of TPP and Mg^{++} or Mn^{++} ions, the resin-treated extracts formed only acetoin from pyruvate—not diacetyl and acetoin, as did untreated extracts—and the ability to form acetoin was retained only if the *p*H during the resin treatment was maintained between 7.0 and 7.3. Subsequently, it was found possible to restore the diacetyl-producing property of resin-treated extracts by adding acetyl-CoA to the reaction mixtures (Fig. 5). Acetyl-phosphate did not replace the acetyl-CoA. Neither diacetyl nor acetoin was formed when the otherwise complete reaction

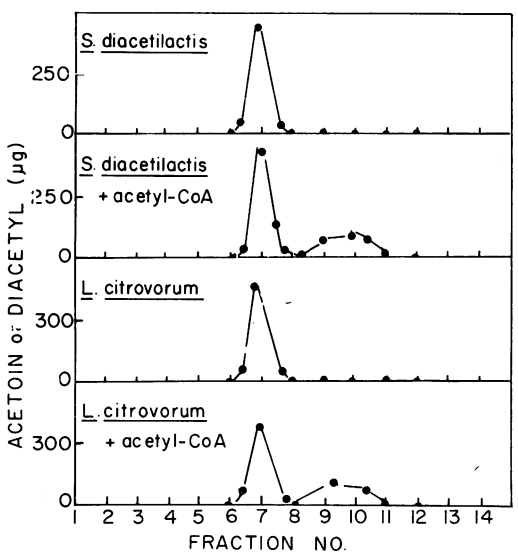

FIG. 5. *Influence of acetyl-CoA on diacetyl biosynthesis by Dowex-1-X2-treated cell-free extracts of Streptococcus diacetilactis and Leuconostoc citrovorum. Reaction mixtures contained: Na-pyruvate, 25 μmoles; TPP, 0.02 μmole; $MgSO_4$, 4.5 μmoles; resin-treated extract protein, 5 mg; 0.05 M phosphate buffer, pH 5.3; total volume, 3.0 ml; temperature, 25 C; with or without acetyl-CoA, 10 μmoles.*

system was modified by using boiled extract or by omitting the extract, pyruvate, TPP, or the metal ions.

The role of acetyl-CoA in diacetyl biosynthesis was confirmed for *S. diacetilactis* in an experiment in which 1 μc of acetyl-CoA-*1*-^{14}C was added to a test system containing 100 μmoles of unlabeled sodium pyruvate, 0.025 μmole of TPP, 4.5 μmoles of Mg^{++} ions, and 3 mg of extract protein, in 0.1 M KH_2PO_4 buffer at *p*H 4.5. The mixture was held for 30 min at 25 C, and a sample was removed for separation of acetoin and diacetyl and examination of each for radioactivity. The resulting diacetyl was found to be highly labeled, with a specific activity of 4.77×10^5 counts per min per μmole. The acetoin, however, was not radioactive, showing that its biosynthesis had not involved acetyl-CoA.

DISCUSSION

S. diacetilactis and *L. citrovorum*, the important diacetyl-producing bacteria used in making cultured products, were found unable to produce diacetyl from α-acetolactate, acetoin, or acetaldehyde. Apparently their only mechanism for producing diacetyl is the mechanism newly indicated by the results reported here, wherein an acetaldehyde-TPP complex formed from pyruvate attacks the carbonyl carbon of acetyl-CoA. By this proposed mechanism (Fig. 6), the thiol-ester linkage provided by the acetyl-CoA moiety allows a migration of electrons to yield diacetyl as the product of rearrangement. Whether other microorganisms use this mechanism, and its relations to other biochemical processes, are being investigated.

These bacteria have two mechanisms for producing acetoin, each involving the intermediate formation of an acetaldehyde-TPP complex from pyruvate. One mechanism is by decarboxylation of α-acetolactate following its formation by condensation of the complex with another molecule of pyruvate. This mechanism, first indicated by Juni (8) to be important in bacteria, is substantiated by the results reported here. Rapid conversion of only about 50% of commercial α-acetolactate to acetoin by cell-free extracts of *S. diacetilactis* and *L. citrovorum* indicates that this enzymatic conversion involves only one of the optically active forms, since the commercial α-acetolactate employed as substrate was racemic. This result distinguishes the acetoin formed by this enzyme from that produced by pyruvic oxidase preparations, which yielded racemic acetoin (10).

The second mechanism used by these bacteria for producing acetoin is by reducing part or all of the diacetyl that is formed from condensation of

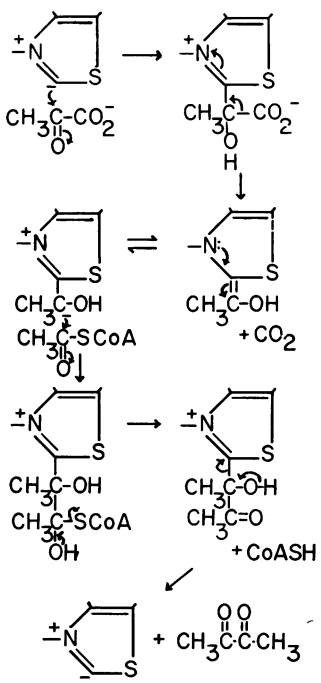

FIG. 6. *Proposed mechanism for diacetyl biosynthesis by Streptococcus diacetilactis and Leuconostoc citrovorum.*

the acetaldehyde-TPP complex with acetyl-CoA. Harvey and Collins (6, 7) pointed out that the production of acetoin in *S. diacetilactis* serves as a detoxification mechanism for removing intracellular pyruvate not required for the synthesis of cell material. Successive reductions of diacetyl to acetoin to 2,3-butylene glycol also appear useful to the bacterial cell. They provide a path for regenerating the cell's NAD supply with the simultaneous formation of only neutral molecules. In contrast, NAD regeneration by glycolysis is tightly coupled to the formation of toxic lactic acid.

In addition to providing evidence for a new mechanism for the biosynthesis of diacetyl, the present results have an important bearing on previous interpretations of results. Failure of the decarboxylation of α-acetolactate to yield detectable diacetyl, failure of acetoin to be involved in the biosynthesis of diacetyl, and the considerable variations that have been observed among bacterial strains and species in ability to reduce diacetyl (24) leave little reason to suppose that there is correlation between the acetoin and diacetyl contents of lactic cultures. These findings enhance the importance of using methods that separate or distinguish between diacetyl and acetoin, and place in question the results of a large number of published papers in which the sum of acetoin and diacetyl has been considered to indicate the production of diacetyl by bacteria.

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LITERATURE CITED

1. COX, G. A. 1945. The effect of acidity on the production of diacetyl by betacocci in milk. J. Dairy Res. **14**:28–35.
2. DOLIN, M. 1955. The DPNH-oxidizing enzymes of *Streptococcus faecalis*. II. Arch. Biochem. Biophys. **55**:415–435.
3. DOLIN, M. I., AND I. C. GUNSALUS. 1951. Pyruvic acid metabolism. II. An acetoin-forming enzyme system in *Streptococcus faecalis*. J. Bacteriol. **62**:199–214.
4. GORNALL, A. G., C. J. BARDSWILL, AND M. M. DAVIS. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177**:751–766.
5. HARVEY, R. J., AND E. B. COLLINS. 1961. Role of citritase in acetoin formation by *Streptococcus diacetilactis* and *Leuconostoc citrovorum*. J. Bacteriol. **82**:954–959.
6. HARVEY, R. J., AND E. B. COLLINS. 1962. Factors responsible for the relationship between *p*H and acetoin formation by *Streptococcus diacetilactis*. Intern. Congr. Microbiol., 8th, Montreal, p. 46.
7. HARVEY, R. J., AND E. B. COLLINS. 1963. Roles of citrate and acetoin in the metabolism of *Streptococcus diacetilactis*. J. Bacteriol. **86**:1301–1307.
8. JUNI, E. 1952. Mechanisms of formation of acetoin by bacteria. J. Biol. Chem. **195**:715–726.
9. JUNI, E. 1952. Mechanisms of the formation of acetoin by yeast and mammalian tissue. J. Biol. Chem. **195**:727–734.
10. JUNI, E., AND G. A. HEYM. 1956. Acyloin condensation reactions of pyruvic oxidase. J. Biol. Chem. **218**:365–378.
11. KEENAN, T. W., R. C. LINDSAY, M. E. MORGAN, AND E. A. DAY. 1966. Acetaldehyde production by single-strain lactic streptococci. J. Dairy Sci. **49**:10–14.
12. KOSIKOWSKI, F. 1966. Cheese and fermented milk foods. Edwards Bros., Ann Arbor, Mich.
13. DE LEY, J. 1959. On the formation of acetoin by *Acetobacter*. J. Gen. Microbiol. **21**:352–365.
14. LINDSAY, R. C., E. A. DAY, AND W. E. SANDINE. 1965. Green flavor defect in lactic starter cultures. J. Dairy Sci. **48**:863–869.
15. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
16. DE MAN, J. C. 1959. The formation of diacetyl and acetoin from α-acetolactic acid. Rec. Trav. Chim. **78**:480–486.

17. MICHAELIAN, M. B., AND B. W. HAMMER. 1936. The oxidation of acetylmethylcarbinol to diacetyl in butter cultures. Iowa Agr. Expt. Sta. Res. Bull. 205.
18. MIZUNO, W. G., AND J. J. JEZESKI. 1959. Starter metabolism. IV. Effects of various substrates on the formation of acetoin by a mixed strain starter culture. J. Dairy Sci. **42**:251–263.
19. VAN NIEL, C. B., A. J. KLUYVER, AND H. G. DERX. 1929. Über das Butteraroma. Biochem. Z. **210**:234–251.
20. PETTE, J. W. 1949. Some aspects of the butter aroma problem. 12th Intern. Dairy Congr. **2**:572–579.
21. Sadtler Research Laboratories. 1959–65. The Sadtler standard spectra. Midget ed., Philadelphia.
22. SEBEK, O. K., AND C. I. RANDLES. 1952. The oxidation of the stereoisomeric 2,3-butanediols by *Pseudomonas*. Arch. Biochem. Biophys. **40**:373–379.
23. SEITZ, E. W., W. E. SANDINE, P. R. ELLIKER, AND E. A. DAY. 1963. Studies on diacetyl biosynthesis by *Streptococcus diacetilactis*. Can. J. Microbiol. **9**:431–441.
24. SEITZ, E. W., W. E. SANDINE, P. R. ELLIKER, AND E. A. DAY. 1963. Distribution of diacetyl reductase among bacteria. J. Dairy Sci. **46**: 186–189.
25. SPECKMAN, R. A., AND E. B. COLLINS. 1968. Separation of diacetyl, acetoin, and 2,3-butylene glycol by salting-out chromatography. Anal. Biochem., *in press*.
26. STRECKER, H. J., AND I. HARARY. 1954. Bacterial butylene glycol dehydrogenase and diacetyl reductase. J. Biol. Chem. **211**:263–270.
27. SUOMALAINEN, H., AND T. LINNAHALME. 1966. Metabolites of α-ketomonocarboxylic acids formed by dried bakers and brewers yeast. Arch. Biochem. Biophys. **114**:502–513.
28. WESTERFELD, W. W. 1945. A colorimetric determination of blood acetoin. J. Biol. Chem. **161**:495–502.